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(54) Gene delivery vectors with cell type specificity for mesenchymal stem cells

(57) The present invention provides novel methods and means for delivering a heterologous nucleic acid of interest to mesenchymal stem cells by providing recombinant adenoviral vectors provided with, or having a natural tropism for mesenchymal stem cells, typically in combination with a reduced tropism for other kinds of cells, in particular liver cells. The invention also provides mesenchymal stem cells provided with a heterologous nucleic acid through the use of a recombinant adenoviral vector according to the invention, and the use of such mesenchymal stem cells for the preparation of medicaments for the treatment of multiple sclerosis, rheumatoid arthritis, angiogenesis and bone related disorders, for instance in treatments that involve bone (re)generation.

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Description

FIELD OF THE INVENTION

[0001] The present invention relates to methods and means for transducing mesenchymal stem cells with a desired nucleic acid. Mesenchymal stem cells are a recently discovered kind of stem cells for which suitable transfer vehicles are still desired. Typical gene delivery vehicles such as the generally used adenoviruses or adeno associated viruses have no particular tropism for mesenchymal stem cells as disclosed herein.
Thus, in particular the present invention relates to the field of gene therapy, more in particular to gene therapy using adenoviruses provided with tropism for mesenchymal stem cells.

BACKGROUND OF THE INVENTION

[0002] In gene therapy, genetic information is delivered to a host cell in order to either correct (supplement) a genetic deficiency in said cell, or to inhibit an unwanted function in said cell, or to eliminate said host cell. Of course the genetic information can also be intended to provide the host cell with a wanted function, for instance to supply a secreted protein to treat other cells of the host, etc.
Thus there are basically three different approaches in gene therapy, one directed towards compensating a deficiency present in a (mammalian) host; the second directed towards the removal or elimination of unwanted substances (organisms or cells) and the third towards providing a cell with a wanted function.
In order to provide cells with a gene (nucleic acid) of interest for any of the purposes identified above, a vehicle capable of delivering said gene to a host cell in an functional format is necessary. In certain instances transient expression (up to a number of weeks or somewhat longer) is desired, in others permanent transduction of a host cell seems necessary.
In order to achieve these goals various vehicles are available. Retroviruses and adeno associated viruses are capable of integrating their genome (including a gene (nucleic acid) of interest) into the genome of a host cell, adenoviruses remain episomal.
However, adenoviruses are better capable of infecting many kinds of cells, whereas retroviruses can only infect certain specific cells. Combinations of different viruses (chimaeric viruses) have been proposed to use the advantages of different kinds of viruses.
Of course non viral delivery systems are also available as are synthetic viruses, all of which can also be adapted to the specific target at hand. All of these systems may be applied in the present invention.
It is known that stem cells are typically difficult to infect to a significant extent with the gene delivery vehicles of the prior art. If infection succeeds, functional expression, especially over a significant amount of time has been difficult to achieve. The infection of stem cells has been considered one of the more disappointing aspects of gene therapy.
The present invention provides methods and means for delivering genes (nucleic acids of interest) to mesenchymal stem cells by providing gen delivery vehicles with a tropism for mesenchymals stem cells, typically in combination with a reduced tropism for other kinds of cells, in particular liver cells.
Thus the invention provides a nucleic acid delivery vehicle having at least a tissue tropism for mesenchymal stem cells and preferably having at least partially reduced tissue tropism for liver cells and other cells with which the gene delivery vehicle may come in contact in the host. Typically said vehicle according to the invention is provided with said tissue tropism by at least a part of a virus capsid (or envelope) or a functional derivative and/or analogue thereof. The capsid may comprise proteins, or functional parts, derivatives and/or analogues thereof from one or more viruses of the same species, but different subtypes or from different viruses. Preferably at least one of said viruses is an adenovirus, typically an adenovirus of subgroup B. Typically at least one of said proteins derived from said capsid comprises a tissue tropism determining part of a fiber protein derived from a subgroup B adenovirus, in particular from an adenovirus of serotype 11, 16, 35 and/or 51 or a functional derivative and/or analogue thereof. The present invention shows that gene delivery vehicles according to the invention provided with a tissue tropism determining part of an adenovirus type B fiber have increased tropism for mesenchymal stem cells. A particular suitable tropism determining part can be derived from the fiber protein of adenovirus 16. It is preferred to use a whole fiber protein of an adenovirus comprising such a part of the fiber of adenovirus 16.
We have found that chimaeric adenoviruses are particularly suitable for targeting mesenchymal stem cells. Thus the invention further provides a gene delivery vehicle according to the invention further comprising at least one protein derived from an adenovirus not belonging to subgroup B, or a functional part, derivative and/or analogue thereof. Preferably said protein or a functional part, derivative and/or analogue thereof not derived from an adenovirus of subgroup B is derived from an adenovirus of subgroup C, preferably of adenovirus 5 for reasons described below. Also the nucleic acid comprising the nucleic acid of interest (the gene) to be delivered is preferably of adenoviral origin, be it of one or more different adenoviruses. Typically the adenovirus derived nucleic acid encodes a fiber protein comprising at least a tissue tropism determining part of a subgroup B adenovirus fiber protein, in particular of a serotype 11, 16,

35 and/or 51, preferably of adenovirus 16 or a functional derivative and/or analogue thereof. Functional derivatives and analogues when used herein are intended to read on molecules based on the ones disclosed herein, be it chemically derived, theoretically derived or experimentally designed to have the same function (in kind not necessarily in amount). This includes in the case of nucleic acids and/or proteinaceous molecules, molecules having a high homology to the particular ones disclosed (both fragments and derivatives) and providing similar function. In some embodiments the nucleic acid delivery vehicle must not replicate in target cells. In such instances it is preferred that said adenovirus nucleic acid is a modified nucleic acid such that the capacity of said adenovirus nucleic acid to replicate in a target cell has been reduced or disabled, preferably through a deletion of at least part of the adenoviral E1-region. In order to achieve expression of the gene of interest over a significant amount of time, it is preferred to provide a gene delivery vehicle capable of avoiding an immune response to a certain extent. Therefor the invention provides a vehicle according to the invention, wherein said adenovirus nucleic acid is a modified nucleic acid such that the capacity of a host immune system to mount an immune response against adenovirus proteins encoded by said adenovirus nucleic acid has been reduced or disabled, preferably through a deletion of adenovirus E2A and/or at least part of the E4-region.

The invention also provides a vehicle according the invention, comprising a minimal adenovirus vector or an Ad/AAV chimaeric vector.

The invention further provides methods for the production of a vehicle according to the invention, comprising providing a cell with means for the assembly of said vehicle wherein said means includes a means for the production of an adenovirus fiber protein, wherein said fiber protein comprises at least a tissue tropism determining part of a subgroup B adenovirus, in particular a serotype 11, 16, 35 and/or 51 adenovirus fiber protein or a functional derivative and/or analogue thereof. Further details are given in the detailed description.

The invention further provides a cell for the production of a vehicle according to the invention, comprising means for the assembly of said vehicle wherein said means includes a means for the production of an adenovirus fiber protein, wherein said fiber protein comprises at least a tissue tropism determining part of a subgroup B adenovirus fiber protein, in particular a serotype 11, 16, 35 and/or 51 adenovirus or a functional derivative and/or analogue thereof, preferably said cell is or is derived from a PER.C6 cell (ECACC deposit number 96022940). Further details are given in the detailed description.

DETAILED DESCRIPTION

[0003] For the sake of brevity the present invention is explained in more detail based on one of its embodiments relating to gene delivery vehicles based on adenoviruses.

For the purpose of gene therapy, adenoviruses have been proposed as suitable vehicles to deliver genes to the host. Gene-transfer vectors derived from adenoviruses (so-called adenoviral vectors) have a number of features that make them particularly useful for gene transfer. 1) the biology of the adenoviruses is characterized in detail, 2) the adenovirus is not associated with severe human pathology, 3) the virus is extremely efficient in introducing its DNA into the host cell, 4) the virus can infect a wide variety of cells and has a broad host-range, 5) the virus can be produced at high virus titers in large quantities, and 6) the virus can be rendered replication defective by deletion of the early-region 1 (E1) of the viral genome (Brody et al, 1994).

[0004] However, there are still drawbacks associated with the use of adenoviral vectors especially the well-investigated serotypes of subgroup C adenoviruses. These serotypes require the presence of the Coxsackie adenovirus receptor (CAR) on cells for successful infection. Although this protein is expressed by many cells and established cell lines, this protein is absent on many other primary cells and cell lines making the latter cells difficult to infect with serotypes 1, 2, 5, and 6.

[0005] The adenovirus genome is a linear double-stranded DNA molecule of approximately 36000 base pairs. The adenovirus DNA contains identical Inverted Terminal Repeats (ITR) of approximately 90-140 base pairs with the exact length depending on the serotype. The viral origins of replication are within the ITRs exactly at the genome ends.

Most adenoviral vectors currently used in gene therapy have a deletion in the E1 region, where novel genetic information can be introduced. The E1 deletion renders the recombinant virus replication defective (Levrero et al 1991). It has been demonstrated extensively that recombinant adenovirus, in particular serotype 5 is suitable for efficient transfer of genes *in vivo* to the liver, the airway epithelium and solid tumors in animal models and human xenografts in immunodeficient mice (Bout 1996; Blaese et al 1995). Thus, preferred methods for *in vivo* gene transfer into target cells make use of adenoviral vectors as gene delivery vehicles.

[0006] At present, six different subgroups of human adenoviruses have been proposed which in total encompasses 51 distinct adenovirus serotypes. Besides these human adenoviruses an extensive number of animal adenoviruses have been identified (see Ishibashi et al 1983).

[0007] A serotype is defined on the basis of its immunological distinctiveness as determined by quantitative neutralization with animal antisera (horse, rabbit). If neutralization shows a certain degree of cross-reaction between two viruses, distinctiveness of serotype is assumed if A) the hemagglutinins are unrelated, as shown by lack of cross-

reaction on hemagglutination-inhibition, or B) substantial biophysical/ biochemical differences in DNA exist (Francki et al 1991). The nine serotypes identified last (42-51) were isolated for the first time from HIV-infected patients (Hierholzer et al 1988; Schnurr et al 1993; De Jong et al 1999). For reasons not well understood, most of such immuno-compromised patients shed adenoviruses that were rarely or never isolated from immuno-competent individuals (Hierholzer et al 1988 and 1992; Khoo et al 1995; De Jong et al 1999).

[0008] The adenovirus serotype 5 is most widely used for gene therapy purposes. Similar to serotypes 2, 4 and 7, serotype 5 has a natural affiliation towards lung epithelia and other respiratory tissues. In contrast, it is known that, for instance, serotypes 40 and 41 have a natural affiliation towards the gastrointestinal tract. For a detailed overview of the disease association of the different adenovirus serotypes see table 1. The natural affiliation of a given serotype towards a specific organ can either be due to a difference in the route of infection i.e. make use of different receptor molecules or internalization pathways. However, it can also be due to the fact that a serotype can infect many tissues/ organs but it can only replicate in one organ because of the requirement of certain cellular factors for replication and hence clinical disease. At present it is unknown which of the above mentioned mechanisms is responsible for the observed differences in human disease association. However it is known that different adenovirus serotypes can bind to different receptors due to sequence dissimilarity of the capsid proteins i.e. hexon, penton, and fiber protein. For instance, it has been shown that adenoviruses of subgroup C such as Ad2, and Ad5 bind to different receptors as compared to adenoviruses from subgroup B such as Ad3 (Defer et al, 1990). Likewise, it was demonstrated that receptor specificity could be altered by exchanging the Ad3 with the Ad5 knob protein, and vice versa (Krasnykh et al 1996; Stevenson et al 1995 and 1997).

[0009] The present invention makes use of the observation that different adenovirus serotypes have different human disease association which might be the result of differences in cellular host range which in turn might be the result of differences in capsid proteins, in particular the fiber protein.

The invention also provides a library of adenoviruses in which the sequence encoding for the fiber protein from alternative serotypes has been cloned into an adenovirus serotype 5 backbone thereby generating a chimaeric adenovirus. This chimaeric adenovirus thus has the host range of the adenovirus serotype of which the fiber sequence was cloned whereas all other aspects are derived from adenovirus serotype 5.

Of course also a gene of interest can be inserted at for instance the site of E1 of the original adenovirus from which the vector is derived. In this manner the chimaeric adenovirus to be produced can be adapted to the requirements and needs of certain hosts in need of gene therapy for certain disorders. Naturally, to enable production of a chimaeric adenovirus, a packaging cell will generally be needed in order to produce sufficient amount of safe chimaeric adenoviruses.

[0010] An important feature of the present invention is the means to produce the chimaeric virus. Typically, one does not want an adenovirus batch to be administered to a host cell which contains replication competent adenovirus, although this is not always true. In general therefor it is desired to omit a number of genes (but at least one) from the adenoviral genome on the vector encoding the chimaeric virus and to supply these genes in the genome of the cell in which the vector is brought to produce chimaeric adenovirus. Such a cell is usually called a packaging cell. The invention thus also provides a packaging cell for producing a chimaeric adenovirus according to the invention, comprising in trans all elements necessary for adenovirus production not present on the adenoviral vector according to the invention. Typically vector and packaging cell have to be adapted to one another in that they have all the necessary elements, but that they do not have overlapping elements which lead to replication competent virus by recombination.

In the human body circulating blood cells survive for a predetermined period which, depending on the cell type can vary from hours to months. Therefore, there is a continuous need for red cells, platelets, lymphocytes, monocytes, and granulocytes. These cell pools are kept at a constant level through division and differentiation of hemopoietic stem cells (HSCs) derived from bone marrow. One of the features of HSCs is that they not adhere to plastic culture dishes under ex vivo culture conditions. Besides HSC, in recent years another stem-cell like cell has been identified in bone marrow which is of non-hemopoietic origin and which does adhere to plastic support. This stem cell-like cell is of mesenchymal origin and is therefore designated as mesenchymal stem cells or bone marrow marrow stromal cells (MSCs). Many attributes of MSCs remain to be determined but their multipotentiality is demonstrated by the observations that MSCs can differentiate into osteoclasts, chondroblasts, fibroblasts, adipocytes and myoblasts (reviewed: Prockop et al 1997). Although HSCs and MSCs can be isolated from bone marrow, only the latter can be derived from other tissues such as skeletal muscle (Jackson et al 1999), liver (Crosbie et al 1999), and brain (Bjornson et al 1999). The pluripotency of MSCs was futher emphizised by the observation that MSCs derived from brain were capable to produce a variety of blood cell types including myeloid and lymphoid cells (Bjornson et al 1999). Like HSCs, the MSCs are being explored as vehicles for cell-, and gene therapy. MSCs are relatively easy to obtain from small bone marrow aspirates under local anesthesia and are easy to culture under *ex vivo* conditions without loosing their stem cell characteristics. Applications of MSCs for therapeutic interventions are both *ex vivo*, in the field of tissue engineering, as well as *in vivo* for treatment of acquired or congenital diseases. One, non-limiting, example is to isolate MSCs from the bone marrow of patients afflicted with degenerative arthritis, expand the cells in culture, genetically modify the cells

such that they express factors which interfere with for instance synoviocyte proliferation or inflammation, and then transplant the cells directly into the afflicted joints. Non-limiting examples of genes which interfere either with synoviocyte proliferation or inflammation is IL-10 (Dechanet et al 1995) or soluble VCAM-1 (Chen et al 1995). Another application is the implantation of MSCs genetically modified to express stimulators of bone regeneration. A non-limiting example of such genes is bone morphogenesis protein-2 (BMP-2) or LIM mineralization protein-1 (LMP-1) (Lou et al 1999, Boden et al 2000). These genetically modified MSCs are subsequently transplanted locally to induce bone formation. Alternatively, the cells are cultured ex vivo in a bio-reactor on a collagen scaffold to form artificial bone that can be surgically transplanted. Locally injected MSCs were shown to promote repair of surgical incisions in the knee cartilage of rabbits, and MSCs in ceramic beads were shown to promote bone healing in an animal model (Goldberg et al 1994, Bruder et al 1994, Wakitani et al 1994). The same strategy can be applied to treat for instance multiple sclerosis. Here, autologous MSCs are genetically modified to express dystrophin before transplantation into striated muscle. Another example is to treat heart failure in which case MSCs expressing angiogenic inducing factors are transplanted in ischemic regions of a diseased heart. In the latter case genes such as nitric oxide synthase (NOS1-3), vascular endothelial growth factor (VEGF), or C-naturetic peptide (CNP) can be used.

[0011] Another, non-limiting, example of an application of MSCs is to infuse MSCs systemically after the cells have been genetically modified to express secreted proteins. The MSCs home at different places in the body secreting a therapeutic protein that exerts its function at specific (distant) sites. Non-limiting examples of interesting genes are factor VIII, factor IX, or mutant genes for β-glucocerebrosidase, erythropoietin (EPO), α-L-iduronidase, iduronate sulphatase, N-sulphatase, N-acetyl α-D-glucosaminidase, α-glucosamine-N-acetyltransferase, N-acetyl-α-D-glucosaminide-6-sulphatase, Galactosamine-6 sulphate sulphatase, β-galactosidase, N-acetyl-alactosamine-4-sulphatase, acid ceraminidase, acid sphingomyelinase, galactocerebroside β-galactosidase, arylsuphatase A, adenosine deaminase, α-L-fucosidase growth factors such as the interleukin family, angiogenesis stimulating or inhibiting factors such as the nitric oxide synthases (NOS1-3), vascular endothelial growth factors (VEGF), Angiostatin 1-7. Besides the direct infusion of genetically modified MSCs the cells can encapsulated in inert material that allows the diffusion of proteins, but not the cells. At least for factor IX the feasibility of this approach was shown in immuno-deficient mice transplanted with human MSCs. These mice expressed Factor IX for at least 8 months after systemic infusion.

[0012] One major advantage of MSCs over HSCs is that MSCs can be cultured to large numbers under *ex vivo* conditions without the loss of multipotency. The latter indicates that the abovementioned strategies can be applied to patients without the need for marrow ablative conditioning simply because large numbers of genetically modified cells can be infused. The examples described indicate that MSCs are of interest for many different therapeutic strategies and therefore genetic modification of this cell type is of considerable importance. For many applications transient expression of exogenous genes is sufficient to trigger the MSCs to differentiate or to express periodically secreted proteins. Transient expression is sufficient if a process needs to be triggered i.e. angiogenesis, chondrogenesis, bone formation. For other applications sustained expression is necessary i.e. protein secretion to elevate protein deficiency (Factor VIII, Factor IX, sugar reducing enzymes etc). For all applications adenoviruses as gene delivery vehicle can be used, since an adenovirus can be engineered to either or not integrate into the host cell genome (Conçalves et al; 2000). Alternative strategies to obtain integration and thus long-term expression have been published (Feng et al 1997; Zheng et al 2000). In these studies an adenovirus is used to deliver an integrating vector (retrovirus) by cloning the retrovirus genome into the adenovirus genome. To identify an adenovirus which efficiently transduces MSCs provides clinical applications of any of the above mentioned strategies. Therefore, it is important to understand the molecular basis of adenovirus binding and internalization. For this reason, the steps involved in adenovirus binding as has been elucidated at present for adenovirus serotype 5 will be described. The initial step for successful infection is binding of the adenovirus to its target cell, a process mediated through fiber protein. The fiber protein has a trimeric structure (Stouten et al 1992) with different lengths depending on the virus serotype (Signas et al 1985; Kidd et al 1993). Different serotypes have polypeptides with structurally similar N and C termini, but different middle stem regions. N-terminally, the first 30 amino acids are involved in anchoring of the fiber to the penton base (Chroboczek et al 1995), especially the conserved FNPVYP region in the tail (Arnberg et al 1997). The C-terminus, or knob, is responsible for initial interaction with the cellular adenovirus receptor. After this initial binding secondary binding between the capsid penton base and cell-surface integrins is proposed to lead to internalisation of viral particles in coated pits and endocytosis (Morgan et al 1969; Svensson et al 1984; Varga et al 1992; Greber et al 1993; Wickham et al 1995). Integrins are αβ-heterodimers of which at least 14 α-subunits and 8 β-sububits have been identified (Hynes et al 1992). The array of integrins expressed in cells is complex and will vary between cell types and cellular environment. Although the knob contains some conserved regions, between serotypes, knob proteins show a high degree of variability, indicating that different adenovirus receptors might exist. For instance, it has been demonstrated that adenoviruses of subgroup C (Ad2, Ad5) and adenoviruses of subgroup B (Ad3) bind to different receptors (Defer et al 1990). By using baculovirus produced soluble CAR as well as adenovirus serotype 5 knob protein, Roelvink et al (1998) concluded via interference studies that all adenovirus serotypes, except serotypes of subgroup B, enter cells via CAR.

[0013] Besides the involvement in cell binding, the fiber protein also contains the type specific γ-antigen, which to-

gether with the ε-antigen of the hexon determines the serotype specificity. The γ-antigen is localized on the fiber and it is known that it consists of 17 amino acids. The anti-fiber antibodies of the host are therefore directed to the trimeric structure of the knob. To obtain re-directed infection of recombinant adenovirus serotype 5, several approaches have been or still are under investigation. Wickham et al (1993 and 1995) have altered the RGD (Arg, Gly, Asp) motif in the penton base which is believed to be responsible for αvβ3 and/or αvβ5 integrin binding to the penton base. They have replaced this RGD motif by another peptide motif that is specific for the α4β1 receptor. In this way targeting the adenovirus to a specific target cell could be accomplished. Krasnykh et al (1998) have made use of the HI loop available in the knob. This loop is, based on X-ray crystallographics, located on the outside of the knob trimeric structure and therefore is thought not to contribute to the intramolecular interactions in the knob. Insertion of a FLAG coding sequence into the HI loop resulted in targeting of the adenovirus to target cells by using antibodies which recognize both the FLAG epitope and a cellular receptor (Krasnykh et al 1998). However, complete CAR independent infection was not observed.

[0014] It is an object of the present invention to provide a method and means by which an adenovirus can infect primary human mesenchymal stem cells. Therefore, in one embodiment the generation of chimaeric adenoviruses based on adenovirus serotype 5 with modified fiber genes is described. For this purpose, two or three plasmids, which together contain the complete adenovirus serotype 5 genome, were constructed. From one of these plasmids the DNA encoding the adenovirus serotype 5 fiber protein was removed and replaced by linker DNA sequences that facilitate easy cloning. The plasmid in which the native adenovirus serotype 5 fiber sequence was partially removed subsequently served as template for the insertion of DNA encoding for fiber protein derived from different adenovirus serotypes (human or animal). The DNAs derived from the different serotypes were obtained using the polymerase chain reaction technique in combination with (degenerate) oligo-nucleotides. At the former E1 location in the genome of adenovirus serotype 5, any gene of interest can be cloned. A single transfection procedure of the two or three plasmids together resulted in the formation of a recombinant, fiber chimaeric adenovirus. Although successful introduction of changes in the adenovirus serotype 5 fiber and penton-base have been reported by others, the complex structure of knob and the limited knowledge of the precise aminoacids interacting with CAR render such targeting approaches laborious and difficult.

To overcome the limitations described above we preferably used pre-existing adenovirus fibers to maximize the chance of obtaining recombinant adenovirus which can normally assemble in the nucleus of a producer cell and which can be produced on pre-existing packaging cells. By generating a chimaeric adenovirus serotype 5 based fiber library containing fiber proteins of all other human adenovirus serotypes, we have developed a technology which enables rapid screening for a recombinant adenoviral vector with preferred infection characteristics for primary human mesenchymal stem cells.

[0015] In one aspect the invention describes the construction and use of plasmids consisting of distinct parts of adenovirus serotype 5 in which the gene encoding for fiber protein has been replaced with DNA derived from alternative human or animal serotypes. This set of constructs, in total encompassing the complete adenovirus genome, allows for the construction of unique chimaeric adenoviruses customised for transduction of particular cell types or organ(s). Also, in this part of the invention means and methods to propagate, produce, and purify fiber chimaeric adenoviruses is described.

[0016] In another aspect of the invention chimaeric viruses are described which have preferred infection characteristics in human mesenchymal stem cells. The adenoviral vectors preferably are derived from subgroup B adenoviruses or contain at least a functional part of the fiber protein from an adenovirus from subgroup B comprising at least the binding moiety of the fiber protein. In a further preferred embodiment the adenoviral vectors are chimaeric vectors based on adenovirus serotpye 5 and contain at least a functional part of the fiber protein from adenovirus type 16, 35, or 51. It is to be understood that in all embodiments the adenoviral vectors may be derived from the serotype having the desired properties or that the adenoviral vector is based on an adenovirus from one serotype and contains the sequences comprising the desired functions of another serotype, these sequences replacing the native sequences in the said serotype

[0017] In another aspect of the invention the chimaeric adenoviruses may, or may not, contain deletions in the E1 region and insertions of heterologous genes linked either or not to a promoter. Furthermore, chimaeric adenoviruses may, or may not, contain deletions in the E3 region and insertions of heterologous genes linked to a promoter. Furthermore, chimaeric adenoviruses may, or may not, contain deletions in the E2 and/ or E4 region and insertions of heterologous genes linked to a promoter. In the latter case E2 and/ or E4 complementing cell lines are required to generate recombinant adenoviruses.

EXAMPLES

Example 1: Generation of adenovirus serotype 5 genomic plasmid clones

[0018] The complete genome of adenovirus serotype 5 has been cloned into various plasmids or cosmids to allow easy modification of parts of the adenovirus serotype 5 genome, while still retaining the capability to produce recombinant virus. For this purpose the following plasmids were generated:

1. pBr/Ad.Bam-rITR (ECACC deposit P97082122)

[0019] In order to facilitate blunt end cloning of the ITR sequences, wild-type human adenovirus type 5 (Ad5) DNA was treated with Klenow enzyme in the presence of excess dNTPs. After inactivation of the Klenow enzyme and purification by phenol/chloroform extraction followed by ethanol precipitation, the DNA was digested with BamHI. This DNA preparation was used without further purification in a ligation reaction with pBr322 derived vector DNA prepared as follows: pBr322 DNA was digested with EcoRV and BamHI, dephosphorylated by treatment with TSAP enzyme (Life Technologies) and purified on LMP agarose gel (SeaPlaque GTG). After transformation into competent *E. coli* DH5a (Life Techn.) and analysis of ampiciline resistant colonies, one clone was selected that showed a digestion pattern as expected for an insert extending from the BamHI site in Ad5 to the right ITR.
Sequence analysis of the cloning border at the right ITR revealed that the most 3' G residue of the ITR was missing, the remainder of the ITR was found to be correct. Said missing G residue is complemented by the other ITR during replication.

2. pBr/Ad.Sal-rITR (ECACC deposit P97082119)

[0020] pBr/Ad.Bam-rITR was digested with BamHI and SalI. The vector fragment including the adenovirus insert was isolated in LMP agarose (SeaPlaque GTG) and ligated to a 4.8 kb SalI-BamHI fragment obtained from wt Ad5 DNA and purified with the Geneclean II kit (Bio 101, Inc.). One clone was chosen and the integrity of the Ad5 sequences was determined by restriction enzyme analysis. Clone pBr/Ad.Sal-rITR contains adeno type 5 sequences from the SalI site at bp 16746 up to and including the rITR (missing the most 3' G residue).

3. pBr/Ad.Cla-Bam (ECACC deposit P97082117)

[0021] wt Adeno type 5 DNA was digested with ClaI and BamHI, and the 20.6 kb fragment was isolated from gel by electro-elution. pBr322 was digested with the same enzymes and purified from agarose gel by Geneclean. Both fragments were ligated and transformed into competent DH5a. The resulting clone pBr/Ad.Cla-Bam was analysed by restriction enzyme digestion and shown to contain an insert with adenovirus sequences from bp 919 to 21566.

4. pBr/Ad.AflII-Bam (ECACC deposit P97082114)

[0022] Clone pBr/Ad.Cla-Bam was linearized with EcoRI (in pBr322) and partially digested with AflII. After heat inactivation of AflII for 20' at 65°C the fragment ends were filled in with Klenow enzyme. The DNA was then ligated to a blunt double stranded oligo linker containing a PacI site (5'-AATTGTCTTAATTAACCGCTTAA-3'). This linker was made by annealing the following two oligonucleotides: 5'-AATTGTCTTAATTAACCGC-3' and 5'-AATTGCGGTTAATTAAGAC-3', followed by blunting with Klenow enzyme. After precipitation of the ligated DNA to change buffer, the ligations were digested with an excess PacI enzyme to remove concatameres of the oligo. The 22016 bp partial fragment containing Ad5 sequences from bp 3534 up to 21566 and the vector sequences, was isolated in LMP agarose (SeaPlaque GTG), religated and transformed into competent DH5a. One clone that was found to contain the PacI site and that had retained the large adeno fragment was selected and sequenced at the 5' end to verify correct insertion of the PacI linker in the (lost) AflII site.

5. pBr/Ad.Bam-rITRpac#2 (ECACC deposit P97082120) and pBr/Ad.Bam-rITR#8 (ECACC deposit P97082121)

[0023] To allow insertion of a PacI site near the ITR of Ad5 in clone pBr/Ad.Bam-rITR about 190 nucleotides were removed between the ClaI site in the pBr322 backbone and the start of the ITR sequences. This was done as follows: pBr/Ad.Bam-rITR was digested with ClaI and treated with nuclease Bal31 for varying lengths of time (2', 5', 10' and 15'). The extent of nucleotide removal was followed by separate reactions on pBr322 DNA (also digested at the ClaI site), using identical buffers and conditions. Bal31 enzyme was inactivated by incubation at 75°C for 10 minutes, the DNA was precipitated and resuspended in a smaller volume of TE buffer. To ensure blunt ends, DNAs were further

treated with T4 DNA polymerase in the presence of excess dNTPs. After digestion of the (control) pBr322 DNA with SalI, satisfactory degradation (~150 bp) was observed in the samples treated for 10' or 15'. The 10' or 15' treated pBr/Ad.Bam-rITR samples were then ligated to the above described blunted PacI linkers (See pBr/Ad.AflII-Bam). Ligations were purified by precipitation, digested with excess PacI and separated from the linkers on an LMP agarose gel. After religation, DNAs were transformed into competent DH5a and colonies analyzed. Ten clones were selected that showed a deletion of approximately the desired length and these were further analyzed by T-track sequencing (T7 sequencing kit, Pharmacia Biotech). Two clones were found with the PacI linker inserted just downstream of the rITR. After digestion with PacI, clone #2 has 28 bp and clone #8 has 27 bp attached to the ITR.

pWE/Ad.AflII-rITR (ECACC deposit P97082116)

[0024] Cosmid vector pWE15 (Clontech) was used to clone larger Ad5 inserts. First, a linker containing a unique PacI site was inserted in the EcoRI sites of pWE15 creating pWE.pac. To this end, the double stranded PacI oligo as described for pBr/Ad.AflII-BamHI was used but now with its EcoRI protruding ends. The following fragments were then isolated by electro-elution from agarose gel: pWE.pac digested with PacI, pBr/AflII-Bam digested with PacI and BamHI and pBr/Ad.Bam-rITR#2 digested with BamHI and PacI. These fragments were ligated together and packaged using 1 phage packaging extracts (Stratagene) according to the manufacturer's protocol. After infection into host bacteria, colonies were grown on plates and analyzed for presence of the complete insert. pWE/Ad.AflII-rITR contains all adenovirus type 5 sequences from bp 3534 (AflII site) up to and including the right ITR (missing the most 3' G residue).

pBr/Ad.1ITR-Sal(9.4) (ECACC deposit P97082115)

[0025] Adeno 5 wt DNA was treated with Klenow enzyme in the presence of excess dNTPs and subsequently digested with SalI. Two of the resulting fragments, designated left ITR-Sal(9.4) and Sal(16.7)-right ITR, respectively, were isolated in LMP agarose (Seaplaque GTG). pBr322 DNA was digested with EcoRV and SalI and treated with phosphatase (Life Technologies). The vector fragment was isolated using the Geneclean method (BIO 101, Inc.) and ligated to the Ad5 SalI fragments. Only the ligation with the 9.4 kb fragment gave colonies with an insert. After analysis and sequencing of the cloning border a clone was chosen that contained the full ITR sequence and extended to the SalI site at bp 9462.

pBr/Ad.1ITR-Sal(16.7) (ECACC deposit P97082118)

[0026] pBr/Ad.lITR-Sal(9.4) is digested with SalI and dephosphorylated (TSAP, Life Technologies). To extend this clone upto the third SalI site in Ad5, pBr/Ad.Cla-Bam was linearized with BamHI and partially digested with SalI. A 7.3 kb SalI fragment containing adenovirus sequences from 9462-16746 was isolated in LMP agarose gel and ligated to the SalI-digested pBr/Ad.lITR-Sal(9.4) vector fragment.

pWE/Ad.AflII-EcoRI

[0027] pWE.pac was digested with ClaI and 5' protruding ends were filled using Klenow enzyme. The DNA was then digested with PacI and isolated from agarose gel. pWE/AflII-rITR was digested with EcoRI and after treatment with Klenow enzyme digested with PacI. The large 24 kb fragment containing the adenoviral sequences was isolated from agarose gel and ligated to the ClaI-digested and blunted pWE.pac vector using the Ligation Express™ kit from Clontech. After transformation of Ultracompetent XL10-Gold cells from Stratagene, clones were identified that contained the expected insert. pWE/AflII-EcoRI containes Ad5 sequences from bp 3534-27336.

Construction of new adapter plasmids

[0028] The absence of sequence overlap between the recombinant adenovirus and E1 sequences in the packaging cell line is essential for safe, RCA-free generation and propagation of new recombinant viruses. The adapter plasmid pMLPI.TK is an example of an adapter plasmid designed for use according to the invention in combination with the improved packaging cell lines of the invention. This plasmid was used as the starting material to make a new vector in which nucleic acid molecules comprising specific promoter and gene sequences can be easily exchanged.

[0029] First, a PCR fragment was generated from pZipΔMo+PyF101(N^-) template DNA (described in PCT/NL96/00195) with the following primers: LTR-1: 5'-CTG TAC GTA CCA GTG CAC TGG CCT AGG CAT GGA AAA ATA CAT AAC TG-3' and LTR-2: 5'-GCG GAT CCT TCG AAC CAT GGT AAG CTT GGT ACC GCT AGC GTT AAC CGG GCG ACT CAG TCA ATC G-3'. Pwo DNA polymerase (Boehringer Mannheim) was used according to manufacturers protocol with the following temperature cycles: once 5' at 95°C; 3' at 55°C; and 1' at 72°C, and 30 cycles of 1' at 95°C,

1' at 60°C, 1' at 72°C, followed by once 10' at 72°C. The PCR product was then digested with BamHI and ligated into pMLP10 (Levrero et al 1991) vector digested with PvuII and BamHI, thereby generating vector pLTR10. This vector contains adenoviral sequences from bp 1 up to bp 454 followed by a promoter consisting of a part of the Mo-MuLV LTR having its wild-type enhancer sequences replaced by the enhancer from a mutant polyoma virus (PyF101). The promoter fragment was designated L420. Next, the coding region of the murine HSA gene was inserted. pLTR10 was digested with BstBI followed by Klenow treatment and digestion with NcoI. The HSA gene was obtained by PCR amplification on pUC18-HSA (Kay et al 1990) using the following primers: HSA1, 5'-GCG CCA CCA TGG GCA GAG CGA TGG TGG C-3' and HSA2, 5'-GTT AGA TCT AAG CTT GTC GAC ATC GAT CTA CTA ACA GTA GAG ATG TAG AA-3'. The 269 bp amplified fragment was subcloned in a shuttle vector using the NcoI and BglII sites. Sequencing confirmed incorporation of the correct coding sequence of the HSA gene, but with an extra TAG insertion directly following the TAG stop codon.The coding region of the HSA gene, including the TAG duplication was then excised as a NcoI (sticky)-SalI (blunt) fragment and cloned into the 3.5 kb NcoI(sticky)/BstBI(blunt) fragment from pLTR10, resulting in pLTR-HSA10.

Finally, pLTR-HSA10 was digested with EcoRI and BamHI after which the fragment containing the left ITR, packaging signal, L420 promoter and HSA gene was inserted into vector pMLPI.TK digested with the same enzymes and thereby replacing the promoter and gene sequences. This resulted in the new adapter plasmid pAd/L420-HSA that contains convenient recognition sites for various restriction enzymes around the promoter and gene sequences. SnaBI and AvrII can be combined with HpaI, NheI, KpnI, HindIII to exchange promoter sequences, while the latter sites can be combined with the ClaI or BamHI sites 3' from HSA coding region to replace genes in this construct.

Another adapter plasmid that was designed to allow easy exchange of nucleic acid molecules was made by replacing the promoter, gene and poly A sequences in pAd/L420-HSA with the CMV promoter, a multiple cloning site, an intron and a poly-A signal. For this purpose, pAd/L420-HSA was digested with AvrII and BglII followed by treatment with Klenow to obtain blunt ends. The 5.1 kb fragment with pBr322 vector and adenoviral sequences was isolated and ligated to a blunt 1570 bp fragment from pcDNA1/am (Invitrogen) obtained by digestion with HhaI and AvrII followed by treatment with T4 DNA polymerase. This adapter plasmid was named pCLIP.

Generation of recombinant adenoviruses

[0030] To generate E1 deleted recombinant adenoviruses with the new plasmid-based system, the following constructs are prepared:

a) An adapter construct containing the expression cassette with the gene of interest linearised with a restriction enzyme that cuts at the 3' side of the overlapping adenoviral genome fragment, preferably not containing any pBr322 vector sequences, and

b) A complementing adenoviral genome construct pWE/Ad.AflII-rITR digested with PacI.

These two DNA molecules are further purified by phenol/chloroform extraction and EtOH precipitation. Co-transfection of these plasmids into an adenovirus packaging cell line, preferably a cell line according to the invention, generates recombinant replication deficient adenoviruses by a one-step homologous recombination between the adapter and the complementing construct.

Alternatively, instead of pWE/Ad.AflII-rITR other fragments can be used, e.g., pBr/Ad.Cla-Bam digested with EcoRI and BamHI or pBr/Ad.AflII-BamHI digested with PacI and BamHI can be combined with pBr/Ad.Sal-rITR digested with SalI. In this case, three plasmids are combined and two homologous recombinations are needed to obtain a recombinant adenovirus. It is to be understood that those skilled in the art may use other combinations of adapter and complementing plasmids without departing from the present invention.

A general protocol as outlined below and meant as a non-limiting example of the present invention has been performed to produce several recombinant adenoviruses using various adapter plasmids and the Ad.AflII-rITR fragment. Adenovirus packaging cells (PER.C6) were seeded in 25 cm^2 flasks and the next day when they were at ~80% confluency, transfected with a mixture of DNA and lipofectamine agent (Life Techn.) as described by the manufacturer. Routinely, 40 µl lipofectamine, 4 µg adapter plasmid and 4 µg of the complementing adenovirus genome fragment AflII- rITR (or 2 µg of all three plasmids for the double homologous recombination) are used. Under these conditions transient transfection efficiencies of ~50% (48 hrs post transfection) are obtained as determined with control transfections using a pAd/CMV-LacZ adapter. Two days later, cells are passaged to 80 cm^2 flasks and further cultured. Approximately five (for the single homologous recombination) to eleven days (for the double homologous recombination) later a cytopathogenic effect (CPE) is seen, indicating that functional adenovirus has formed. Cells and medium are harvested upon full CPE and recombinant virus is released by freeze-thawing. An extra amplification step in 80 cm^2 flasks is routinely performed to increase the yield since at the initial stage the titers are found to be variable despite the occurrence of full CPE. After amplification, viruses are harvested and plaque purified on PER.C6 cells. Individual plaques are tested

for viruses with active transgenes.

[0031] Besides replacements in the E1 region it is possible to delete or replace (part of) the E3 region in the adenovirus because E3 functions are not necessary for the replication, packaging and infection of the (recombinant) virus. This creates the opportunity to use a larger insert or to insert more than one gene without exceeding the maximum package size (approximately 105% of wt genome length). This can be done, e.g., by deleting part of the E3 region in the pBr/Ad.Bam-rITR clone by digestion with XbaI and religation. This removes Ad5 wt sequences 28592-30470 including all known E3 coding regions. Another example is the precise replacement of the coding region of gp19K in the E3 region with a polylinker allowing insertion of new sequences. This, 1) leaves all other coding regions intact and 2) obviates the need for a heterologous promoter since the transgene is driven by the E3 promoter and pA sequences, leaving more space for coding sequences.

To this end, the 2.7 kb EcoRI fragment from wt Ad5 containing the 5' part of the E3 region was cloned into the EcoRI site of pBluescript (KS$^-$) (Stratagene). Next, the HindIII site in the polylinker was removed by digestion with EcoRV and HincII and subsequent religation. The resulting clone pBS.Eco-Eco/ad5DHIII was used to delete the gp19K coding region. Primers 1 (5'-GGG TAT TAG GCC AA AGG CGC A-3') and 2 (5'-GAT CCC ATG GAA GCT TGG GTG GCG ACC CCA GCG-3') were used to amplify a sequence from pBS.Eco-Eco/Ad5DHIII corresponding to sequences 28511 to 28734 in wt Ad5 DNA. Primers 3 (5'-GAT CCC ATG GGG ATC CTT TAC TAA GTT ACA AAG CTA-3') and 4 (5'-GTC GCT GTA GTT GGA CTG G-3') were used on the same DNA to amplify Ad5 sequences from 29217 to 29476. The two resulting PCR fragments were ligated together by virtue of the new introduced NcoI site and subsequently digested with XbaI and MunI. This fragment was then ligated into the pBS.Eco-Eco/ad5ΔHIII vector that was digested with XbaI (partially) and MunI generating pBS.Eco-Eco/ad5AHIII.A gp19K. To allow insertion of foreign genes into the HindIII and BamHI site, an XbaI deletion was made in pBS.Eco-Eco/ad5 ΔHIII.Δgp19K to remove the BamHI site in the Bluescript polylinker. The resulting plasmid pBS.Eco-Eco/ad5ΔHIIIΔ gp19KΔXbaI, containes unique HindIII and BamHI sites corresponding to sequences 28733 (HindIII) and 29218 (BamHI) in Ad5. After introduction of a foreign gene into these sites, either the deleted XbaI fragment is re-introduced, or the insert is recloned into pBS.Eco-Eco/ad5ΔHIII. Δgp19K using HindIII and for example MunI. Using this procedure, we have generated plasmids expressing HSV-TK, hIL-1a, rat IL-3, luciferase or LacZ. The unique SrfI and NotI sites in the pBS.Eco-Eco/ad5ΔHIII.Δgpl9K plasmid (with or without inserted gene of interest) are used to transfer the region comprising the gene of interest into the corresponding region of pBr/Ad.Bam-rITR, yielding construct pBr/Ad.Bam-rITRΔgp19K (with or without inserted gene of interest). This construct is used as described *supra* to produce recombinant adenoviruses. In the viral context, expression of inserted genes is driven by the adenovirus E3 promoter. Recombinant viruses that are both E1 and E3 deleted are generated by a double homologous recombination procedure as described above for E1-replacement vectors using a plasmid-based system consisting of:

a) an adapter plasmid for E1 replacement according to the invention, with or without insertion of a first gene of interest,
b) the pWE/Ad.AflII-EcoRI fragment, and
c) the pBr/Ad.Bam-rITRΔgp19K plasmid with or without insertion of a second gene of interest.

In addition to manipulations in the E3 region, changes of (parts of) the E4 region can be accomplished easily in pBr/Ad.Bam-rITR. Generation and propagation of such a virus, however, in some cases demands complementation *in trans*.

Example 2: Generation of adenovirus serotype 5 based viruses with chimaeric fiber proteins

[0032] The method described *infra* to generate recombinant adenoviruses by co-transfection of two, or more seperate cloned adenovirus sequences. One of these cloned adenovirus sequences was modified such that the adenovirus serotype 5 fiber DNA was deleted and substituted for unique restriction sites thereby generating "template clones" which allow for the easy introduction of DNA sequences encoding for fiber protein derived from other adenovirus serotypes.

Generation of adenovirus template clones lacking DNA encoding for fiber

[0033] The fiber coding sequence of adenovirus serotype 5 is located between nucleotides 31042 and 32787. To remove the adenovirus serotype 5 DNA encoding fiber we started with construct pBr/Ad.Bam-rITR. First a NdeI site was removed from this construct. For this purpose, pBr322 plasmid DNA was digested with NdeI after which protruding ends were filled using Klenow enzym. This pBr322 plasmid was then religated, digested with NdeI and transformed into *E.coli* DH5 α. The obtained pBr/ΔNdeI plasmid was digested with ScaI and SalI and the resulting 3198 bp vector fragment was ligated to the 15349 bp ScaI-SalI fragment derived from pBr/Ad.BamrITR, resulting in plasmid pBr/Ad.Bam-rITRΔNdeI which hence contained a unique NdeI site. Next a PCR was performed with oligonucleotides NY-up:

5'- CGA **CAT ATG** TAG ATG CAT TAG TTT GTG TTA TGT TTC AAC GTG-3'
And NY-down: 5'-GGA GAC CAC TGC CAT GTT-3'. During amplification, both a NdeI (bold face) and a NsiI restriction site (underlined) were introduced to facilitate cloning of the amplified fiber DNAs. Amplification consisted of 25 cycles of each 45 sec. at 94°C, 1 min. at 60°C, and 45 sec. at 72°C. The PCR reaction contained 25 pmol of oligonucleotides NY-up or NY-down, 2mM dNTP, PCR buffer with 1.5 mM $MgCl_2$, and 1 unit of Elongase heat stable polymerase (Gibco, The Netherlands). One-tenth of the PCR product was run on an agarose gel which demonstrated that the expected DNA fragment of ± 2200 bp was amplified. This PCR fragment was subsequently purified using Geneclean kit system (Bio101 Inc.). Then, both the construct pBr/Ad.Bam-rITRΔNdeI as well as the PCR product were digested with restriction enzymes NdeI and SbfI. The PCR fragment was subsequently cloned using T4 ligase enzyme into the NdeI and SbfI digested pBr/Ad.Bam-rITRΔNdeI, generating pBr/Ad.BamR_ΔFib. This plasmid allows insertion of any PCR amplified fiber sequence through the unique NdeI and NsiI sites that are inserted in place of the removed fiber sequence. Viruses can be generated by a double homologous recombination in packaging cells described *infra* using an adapter plasmid, construct pBr/Ad.AflII-EcoRI digested with PacI and EcoRI and a pBr/Ad.BamRΔFib construct in which heterologous fiber sequences have been inserted. To increase the efficiency of virus generation, the construct pBr/Ad.BamRΔFib was modified to generate a PacI site flanking the right ITR. Hereto, pBr/Ad.BamRΔFib was digested with AvrII and the 5 kb adenofragment was isolated and introduced into the vector pBr/Ad.Bam-rITR.pac#8 replacing the corresponding AvrII fragment. The resulting construct was named pBr/Ad.BamRΔ Fib.pac. Once a heterologous fiber sequence is introduced in pBr/Ad.BamRΔFib.pac, the fiber modified right hand adenovirus clone may be introduced into a large cosmid clone as described for pWE/Ad.AflII-rITR in example 1. Such a large cosmid clone allows generation of adenovirus by only one homologous recombination making the process extremely efficient.

Amplification of fiber sequences from adenovirus serotypes

[0034] To enable amplification of the DNAs encoding fiber protein derived from alternative serotypes degenerate oligonucleotides were synthesized. For this purpose, first known DNA sequences encoding for fiber protein of alternative serotypes were aligned to identify conserved regions in both the tail-region as well as the knob-region of the fiber protein. From the alignment, which contained the nucleotide sequence of 19 different serotypes representing all 6 subgroups, (degenerate) oligonucleotides were synthesised. The amplification reaction (50 µl) contained 2 mM dNTPs, 25 pmol of each oligonucleotide, standard 1x PCR buffer, 1,5 mM $MgCl_2$, and 1 Unit Pwo heat stable polymerase (Boehringer) per reaction. The cycler program contained 20 cycles, each consisting of 30 sec. 94°C, 60 sec. 60-64°C, and 120 sec. At 72°C. One-tenth of the PCR product was run on an agarose gel which demonstrated that a DNA fragment was amplified. Of each different template, two independent PCR reactions were performed after which the independent PCR fragments obtained were sequenced to determine the nucleotide sequence. From 11 different serotypes, the nucleotide sequence could be compared to sequences present in genbank. Of all other serotypes, the DNA encoding fiber protein was previously unknown and was therefore aligned with known sequences from other subgroup members to determine homology i.e. sequence divergence. Of the 51 human serotypes known to date, all fiber sequences, except for serotypes 1, 6, 18, and 26, have been amplified and sequenced.

Generation of fiber chimaeric adenoviral DNA constructs

[0035] All amplified fiber DNAs as well as the vector (pBr/Ad.BamR ΔFib) were digested with NdeI and NsiI. The digested DNAs was subsequently run on a agarose gel after which the fragments were isolated from the gel and purified using the Geneclean kit (Bio101 Inc). The PCR fragments were then cloned into the NdeI and NsiI sites of pBr/Ad-BamRΔFib, thus generating pBr/AdBamRFibXX (where XX stands for the serotype number of which the fiber DNA was isolated). Sofar the fiber sequence of serotypes 5/ 7/ 8/ 9/ 10/ 11/ 12/ 13/ 14/ 16/17/ 19/ 21/ 24/ 27/ 28/ 29/ 30/ 32/ 33/ 34/ 35/ 36/ 37/ 38/40-S/ 40-L/ 41-S/ 42/45/ 47/ 49/ 51 have been cloned into pBr/AdBamRFibXX. From pBr/AdBamRFibXX (where XX is 5/ 8/ 9/10/ 11/ 13/ 16/ 17/ 24/ 27/ 30/ 32/ 33/ 34/ 35/ 38/ 40-S/40-L/ 45/ 47/ 49/ 51) a cosmid clone in pWE/Ad.AflII-rITR (see example 1) was generated to facilitate efficient virus generaion. This cosmid cloning resulted in the formation of construct pWE/Ad.AflII-rITR/FibXX (where XX stands for the serotype number of which the fiber DNA was isolated)

Generation of recombinant adenovirus chimaeric for fiber protein

[0036] To generate recombinant Ad 5 virus carrying the fiber of serotype 12, 16, 28, 40-L, 51, and 5, three constructs, pCLIP/luciferase, pWE/AdAflII-Eco and pBr/AdBamrITR.pac/fibXX (XX = 12, 16, 28, 40-L, 51, and 5) were transfected into adenovirus producer cells. To generate recombinant Ad5 virus carrying the fiber of 5/ 7/ 8/ 9/ 10/11/ 12/ 13/ 14/ 16/ 17/ 19/ 21/ 24/ 27/ 28/ 29/ 30/ 32/ 33/34/ 35/ 36/ 37/ 38/ 40-S/ 40-L/ 41-S/ 42/45/ 47/ 49/ 51, two contructs pCLIP/ luciferase and pWE/Ad.AflII-rITR/FibXX were transfected into adenovirus producer cells.

For transfection, 2 µg of pCLIP/luciferase, and 4 µg of both pWE/AdAflII-Eco and pBr/AdBamrITR.pac/fibXX (or in case of cosmids: 4 µg of pCLIP/luciferase plus 4 µg of pWE/Ad.AflII-rITR/FibXX) were diluted in serum free DMEM to 100 µl total volume. To this DNA suspension 100 µl 1x diluted lipofectamine (Gibco) was added. After 30 minutes at room temperature the DNA-lipofectamine complex solution was added to 2.5 ml of serum-free DMEM which was subsequently added to a T25 cm^2 tissue culture flask. This flask contained $2x10^6$ PER.C6 cells that were seeded 24-hours prior to transfection. Two hours later, the DNA-lipofectamine complex containing medium was diluted once by the addition of 2.5 ml DMEM supplemented with 20% fetal calf serum. Again 24 hours later the medium was replaced by fresh DMEM supplemented with 10% fetal calf serum. Cells were cultured for 6-8 days, subsequently harvested, and freeze/thawed 3 times. Cellular debri was removed by centrifugation for 5 minutes at 3000 rpm room temperature. Of the supernatant (12.5 ml) 3-5 ml was used to infect again infect PER.C6 cells (T80 cm^2 tissue culture flasks). This re-infection results in full cytopathogenic effect (CPE) after 5-6 days after which the adenovirus is harvested as described above.

Example 3: Production, purification, and titration of fiber chimaeric adenoviruses

[0037] Of the supernatant obtained from transfected PER.C6 cells 10 ml was used to inoculate a 1 liter fermentor which contained 1 - 1.5 x 10^6 cells/ ml PER.C6 that were specifically adapted to grow in suspension. Three days after inoculation, the cells were harvested and pelleted by centrifugating for 10 min at 1750 rpm at roomtemperature. The chimaeric adenoviruses present in the pelleted cells were subsequently extracted and purified using the following downstream proccesing protocol. The pellet was disolved in 50 ml 10 mM $NaPO_4^-$ and frozen at -20°C. After thawing at 37°C, 5.6 ml deoxycolate (5% w/v) was added after which the solution was homogenated. The solution was subsequently incubated for 15 minutes at 37°C to completely crack the cells. After homogenizing the solution, 1875 µl (1M) $MgCl_2$- was added and 5 ml 100% glycerol. After the addition of 375 µl DNAse (10 mg/ ml) the solution was incubated for 30 minutes at 37°C. Cell debri was removed by centrifugation at 1880xg for 30 minutes at room temperature without the brake on. The supernatant was subsequently purified from proteins by loading on 10 ml of freon. Upon centrifugation for 15 minutes at 2000 rpm without brake at room temperature three bands are visable of which the upper band represents the adenovirus. This band was isolated by pipetting after which it was loaded on a Tris/HCl (1M) buffered caesiumchloride blockgradient (range: 1.2 to 1.4 gr./ml). Upon centrifugation at 21000 rpm for 2.5 hours at 10°C the virus was purified from remaining protein and celldebri since the virus, in contrast to the other components, does not migrate into the 1.4 gr./ ml caesiumchloride solution. The virus band is isolated after which a second purification using a Tris/ HCl (1M) buffered continues gradient of 1.33 gr./ml of caesiumchloride is performed. After virus loading on top of this gradient the virus is centrifuged for 17 hours at 55000 rpm at 10°C. Subsequently the virus band is isolated and after the addition of 30 µl of sucrose (50 w/v) excess caesiumchloride is removed by three rounds of dialysis, each round comprising of 1 hour. For dialysis the virus is transferred to dialysis slides (Slide-a-lizer, cut off 10000 kDa, Pierce, USA). The buffers used for dialysis are PBS which are supplemented with an increasing concentration of sucrose (round 1 to 3: 30 ml, 60 ml, and 150 ml sucrose (50% w/v)/ 1.5 liter PBS, all supplemented with 7.5 ml 2% (w/v) $CaMgCl_2$). After dialysis, the virus is removed from the slide-a-lizer after which it is aliquoted in portions of 25 and 100 µl upon which the virus is stored at -85°C.

To determine the number of virus particles per milliliter, 100 µl of the virus batch is run on an high pressure liquid chromatograph (HPLC). The adenovirus is bound to the column (anion exchange) after which it is eluted using a NaCl gradient (range 300-600 mM). By determining the area under the virus peak the number of virus particles can be calculated. To determine the number of infectious units (IU) per ml present in a virus batch, titrations are performed on 911 cells. For this purpose, $4x10^4$ 911 cells are seeded per well of 96-well plates in rows B, D, and F in a total volume of 100 µl per well. Three hours after seeding the cells are attached to the plastic support after which the medium can be removed. To the cells a volume of 200 µl is added, in duplicate, containing different dilutions of virus (range: 10^2 times diluted to $2x10^9$). By screening for CPE the highest virus dilution which still renders CPE after 14 days is considered to contain at least one infectious unit. Using this observation, together with the calculated amount of virus volume present in these wells renders the number of infectious units per ml of a given virus batch. The production results i.e. virus particles per ml and IU per ml or those chimaeric adenoviruses that were produced, all with the luciferase cDNA as a marker, are shown in table 2.

Example 4: Transduction of human mesenchymal stem cells

[0038] The adherent fraction derived from a bone marrow aspirate obtained from a healthy donor was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with D-glucose (1gr/ liter), sodium pyruvate and sodiumbicarbonate (4.4 gr/liter), L-glutamine (2mM), penicillin (100 U/ml), streptomycin (100 µg/ ml), and 10% heat inactivated fetal calf serum. Cells were passaged in a 1:2 ratio upon reaching confluency. For transduction, cells were seeded at a concentration of $5x10^4$ cells per well of 24-well plates, and allowed to adhere for 24 hours. Cells were subsequently

exposed to 1000 virus particles per cell of Ad5 or the fiber chimaeric viruses Ad5.Fib12, Ad5.Fib16, Ad5.Fib32, Ad5.Fib35, Ad5.Fib40-S, Ad5.Fib40-L, Ad5.Fib45, or Ad5.Fib51. The fibers 40-S and 40-L represent the short and the long fiber of serotype 40 respectively. Forty-eight hours after virus addition cells were harvested, and lysed by addition of 100 µl of cell-lysis buffer (PBS/ 1% Triton-X100). Luciferase activity was determined using a bioluminescence machine, the luciferase assay kit from Promega™ (catalog no. E-1501) and the instructions provided by the manufacturer. The results of the luciferase transgene expresssion measured in mesenchymal stem cells after transduction with the panel of fiber chimaeric viruses is shown in figure 1. The results demonstrate that several fiber chimaeric viruses perform better on fibroblasts as compared to the parent vector (Ad5). These viruses carry the fiber from a subgroup B virus i.e. 16, 35, and 51. Also, one subgroup D virus (Ad5.Fib32) seems better equipped for transducing mesenchymal stem cells. Both Ad5.Fib40-S and Ad5.Fib40-L (subgroup F) perform similar or only slightly better as compared to Ad5. In a next experiment we tested the ability of Ad5Fib16 carrying other marker genes to infect mesenchymal stem cells. Hereto, The cells were exposed for two hours to an increasing vector dose of A5 or Ad5Fib16 carrying green fluorescent protein as a marker (Figure 2a). Forty-eight hours later cells were harvested, and using a flow cytometer, the cells were tested for GFP expression. The results obtained demonstrated that, based on the percentage of cells positive for the marker gene, Ad5.Fib16 is much more potent compared to Ad5 for the genetic modification of mesenchymal stem cells. Also, mesenchymal stem cells were seeded in a biodegradable polymeric scafolld and cells were exposed to Ad5.Fib16 carrying LacZ as a marker gene. Again 48 later cells were stained for LacZ expression (figure 2b). Clearly the cells stained blue after transduction with Ad5.Fib16 again showing the efficiency by which Ad5.Fib16 infects Mesenchymal stem cells. Finally, we tested the expression of a marker gene over time to investigate the duration of expression. Hereto, HBSCs were exposed for one hour to 1000 or 500 virus particles per cell of Ad5.Fib16 carrying LacZ. The number of cells scored positive for LacZ was monitored at 24, 48, 72, 120, 144, and 168 hours after the one hour virus exposure. With a dose of 5000 virus particles per cell all cells were infected since 100% of the cells stained positive for LacZ at 48 hours after virus exposure (figure 3a). Generally the time required to obtain optimal expression after infection is 48 hours. Since the number of LacZ positive cells remained at 100% for all time points tested, i.e. until 168 hours after infection it can be concluded that each cell nucleus contains at least 4 copies of the Ad5Fib16 genome since 4 cell doublings did not dilute out the virus. In the latter experiment we also monitored the viability of the transduced cells either infected with 1000 or 5000 virus particles per cell. As control non-transduced cells were analyzed. Base on the percentage of viable cells after transduction with Ad5Fib16, it can be conclude that even a dose of 5000 virus particles per cell does not significantly changes the cellular viability. All together these results show that the improved vectors identified are extremly potent to infect Human bone marow stroma cells or mesenchymal stem cells without compromising the cellular viability. These results thus pave the road to start studies aimed at optimizing the quantity and quality of bioartificial engineered bone

FIGURE AND TABLE LEGENDS

[0039]

Table 1: Association of different human adenovirus serotypes with human disease.

Table 2: Production results of recombinant fiber chimaeric adenoviruses. Results in virus particles per milliliter as determined by HPLC.

Figure 1: Screening the fiber chimaeric viruses for the presence of viruses that are better suited for transduction of primary human mesenchymal stem cells. The dose used is 1000 virus particles per cell. Luciferase activity is expressed (the y-axis) in relative light units (RLU).

Figure 2: Transduction of Human bone marrow stroma cells (MSCs) with Ad5.Fib16. (A): HBSCs were exposed for one hour to 100, 500, or 1000 virus particles of Ad5 or Ad5.Fib16. Forthy-eight hours later cells were screened using a flow cytometer for the expression of GFP. Shown is the average ± standard deviation percentage of cells that are positive for GFP (N=3). (B) HBSCs were exposed to 5000 virus particles perc ell and seeded in a polymeric scaffold. Forty-eight hours after virus exposure the cells were stained for LacZ expression. As a control for the LacZ staining reaction, non-transduced cells (no virus) was taken along.

Figure 3: duration of marker gene expression and toxicity of Ad5Fib16 on mesenchymal stem cells. (A) HBSCs were exposed for one hour to 1000 or 5000 virus particles per cell of Ad5Fib16 lacZ. Cells were stained for LacZ expression at different time points after virus exposure. (B) Cells were infected for one hour with 1000 or 5000 virus particles. At different timepoints after exposure to Ad5Fib16 cells were harvest and the viability of the cell population was determined. As a control cells not exposed (0) to virus were taken along.

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Table 1

Syndrome	Subgenus	Serotype
Respiratory illness	A	31
	B	3, 7, 11, 14, 21, 34, 35, 51
	C	1,2,5,6
	D	39, 42-48
	E	4
Keratoconjunctivitis (eye)	B	11
	D	8, 19, 37, 50
Hemorrhagic cystitis (Kidney) And urogenital tract infections	B	7, 11, 14, 16, 21, 34, 35
	C	5
	D	39, 42-48
Sexual transmission	C	2
	D	19, 37
Gastroenteritis	A	31
	B	3
	C	1, 2, 5
	D	28
	F	40, 41
CNS disease	A	12, 31
	B	3, 7
	C	2, 5, 6
	D	32, 49
Hepatitis	A	31
	C	1,2,5

Table 1 (continued)

Syndrome	Subgenus	Serotype
Disseminated	A	31
	B	3, 7, 11, 21
	D	30, 43-47
None (???)	A	18
	D	9, 10, 13, 15 17, 20, 22-29, 33, 36, 38

Table 2

Adenovirus	Virus particles/ ml
Ad5Fib5	2.2×10^{12}
Ad5Fib9	3.9×10^{11}
Ad5Fib10	5.5×10^{11}
Ad5Fib11	1.1×10^{12}
Ad5Fib12	4.4×10^{12}
Ad5Fib13	1.1×10^{12}
Ad5Fib16	1.4×10^{12}
Ad5Fib17	9.3×10^{11}
Ad5Fib24	1.0×10^{12}
Ad5Fib27	3.0×10^{11}
Ad5Fib30	7.1×10^{11}
Ad5Fib32	2.0×10^{12}
Ad5Fib33	1.5×10^{12}
Ad5Fib35	2.0×10^{12}
Ad5Fib38	5.8×10^{11}
Ad5Fib40-S	3.2×10^{10}
Ad5Fib40-L	2.0×10^{12}
Ad5Fib45	2.8×10^{12}
Ad5Fib47	2.6×10^{12}
Ad5Fib49	1.2×10^{12}
Ad5Fib51	5.1×10^{12}

Claims

1. A nucleic acid delivery vehicle having at least a tissue tropism for mesenchymal stem cells.

2. A nucleic acid delivery vehicle according to claim 1, having at least partially reduced tissue tropism for liver cells.

3. A vehicle according to anyone of claims 1-2, wherein said tissue tropism is provided by at least a part of a virus capsid or a functional derivative and/or analogue thereof.

4. A vehicle according to claim 3, wherein said virus capsid comprises proteins, or functional parts, derivatives and/or analogues thereof, from at least two different viruses.

5. A vehicle according to claim 4, wherein at least one of said viruses is an adenovirus.

6. A vehicle according to claim 4 or claim 5, wherein at least one of said viruses is an adenovirus of subgroup B.

7. A vehicle according to anyone of the claims 4-6, wherein at least one of said proteins comprises a tissue tropism determining part of a fiber protein derived from a subgroup B adenovirus in particular an adenovirus of serotype 11, 16, 35 and/or 51 or a functional derivative and/or analogue thereof.

8. A vehicle according to claim 6 or claim 7, wherein said subgroup B adenovirus is adenovirus 16.

9. A vehicle according to claim 6-8, further comprising at least one protein derived from an adenovirus not belonging to subgroup B, or a functional part, derivative and/or analogue thereof.

10. A vehicle according to claim 9, wherein a protein or a functional part, derivative and/or analogue thereof not derived from an adenovirus of subgroup B is derived from an adenovirus of subgroup C, preferably of adenovirus 5.

11. A vehicle according to anyone of the claims 1-10 comprising nucleic acid derived from an adenovirus.

12. A vehicle according to anyone of the claims 1-11, comprising nucleic acid derived from at least two different adenoviruses.

13. A vehicle according to claim 11 or claim 12, wherein said nucleic acid at least encodes a fiber protein comprising at least a tissue tropism determining part of a subgroup B adenovirus fiber protein, in particular of a serotype 11, 16, 35 and/or 51, preferably of adenovirus 16 or a functional derivative and/or analogue thereof.

14. A vehicle according anyone of claims 11-13, wherein said adenovirus nucleic acid is a modified nucleic acid such that the capacity of said adenovirus nucleic acid to replicate in a target cell has been reduced or disabled, preferably through a deletion of at least part of the E1-region.

15. A vehicle according to anyone of the claims 11-14, wherein said adenovirus nucleic acid is a modified nucleic acid such that the capacity of a host immune system to mount an immune response against adenovirus proteins encoded by said adenovirus nucleic acid has been reduced or disabled, preferably through a deletion of E2A and/or at least part of the E4-region.

16. A vehicle according to anyone of claims 1-15, comprising a minimal adenovirus vector or an Ad/AAV chimaeric vector.

17. A vehicle according to anyone of claims 1-16, further comprising at least one nucleic acid of interest.

18. A vehicle according to anyone of claims 1-17, wherein said vehicle is a subgroup B adenovirus capsid comprising at least one nucleic acid of interest.

19. A vehicle according to claim 18, wherein said nucleic acid further comprises subgroup B adenovirus nucleic acid.

20. A vehicle according to claim 19, wherein said subgroup B adenovirus nucleic acid has been deprived of the capacity to express E1-region encoded proteins.

21. A vehicle according to anyone of claims 18-20, wherein said subgroup B adenovirus is adenovirus 16.

22. A method for the production of a vehicle according to anyone of claims 1-21, comprising providing a cell with means

for the assembly of said vehicle wherein said means includes a means for the production of an adenovirus fiber protein, wherein said fiber protein comprises at least a tissue tropism determining part of a subgroup B adenovirus, in particular a serotype 11, 16, 35 and/or 51 adenovirus fiber protein or a functional derivative and/or analogue thereof.

23. A cell for the production of a vehicle according to anyone of claims 1-21, comprising means for the assembly of said vehicle wherein said means includes a means for the production of an adenovirus fiber protein, wherein said fiber protein comprises at least a tissue tropism determining part of a subgroup B adenovirus fiber protein, in particular a serotype 11, 16, 35 and/or 51 adenovirus or a functional derivative and/or analogue thereof, preferably said cell is or is derived from a PER.C6 cell (ECACC deposit number 96022940).

24. The use of a vehicle according to anyone of claims 1-21 as a pharmaceutical.

25. The use of claim 24 for the treatment of a disease, treatable by transfer of a nucleic acid encoding a therapeutic proteinaceous molecule or RNA to mesenchymal stem cells.

26. The use of nucleic acid delivery vehicle according to anyone of claims 1-22, for the delivery of nucleic acid to mesenchymal stem cells.

27. The use of a vehicle according to anyone of claims 1-22, or a cell according to claim 23, for the generation a nucleic acid library.

28. The use of a fiber protein of adenovirus 16 or a functional part, derivative and/or analogue thereof, for the delivery of nucleic acid to a mesenchymal stem cell.

29. The use of a vehicle according to any one of claims 1-22 in the preparation of a medicament for the treatment of rheumatoid arthritis

30. The use according to claim 29, wherein said vehicle comprises a gene encoding IL-10 or a functional equivalent thereof.

31. The use of a vehicle according to any one of claims 1-22 in tissue engineering.

32. The use of a gene delivery vehicle according to any one of claims 1-22 in the preparation of a medicament for providing bone regeneration.

33. The use of a mesenchymal stem cell provided with a gene of interest through a gene delivery vehicle according to any one of claims 1-22 in the preparation of a medicament for providing bone regeneration.

34. The use according to claim 32 or 33 wherein said gene delivery vehicle is provided with a gene encoding bone morphogenesis protein-2 and/or LIM mineralisation protein-1 or a functional equivalent of either.

35. The use of a mesenchymal stem cell provided with a gene of interest through a gene delivery vehicle according to any one of claims 1-22 in the preparation of a medicament for the treatment of multiple sclerosis.

36. The use of a mesenchymal stem cell provided with a gene of interest through a gene delivery vehicle according to any one of claims 1-22 in the preparation of a medicament for promoting angiogenesis.

37. A mesenchymal stem cell comprising a nucleic acid delivered to said mesenchymal stem cell through a nucleic acid delivery vehicle according to anyone of claims 1-21.

Figure 1

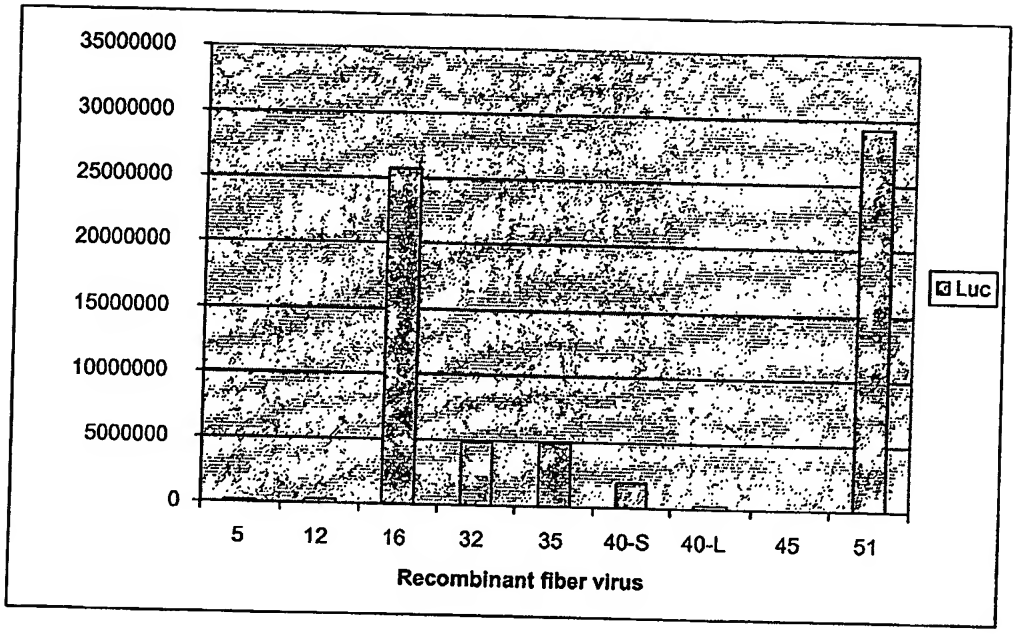

35000000
30000000
25000000
20000000
15000000
10000000
5000000
0
5
12
16
32
35
40-S
40-L
45
51
Recombinant fiber virus
Luc

Figure 2

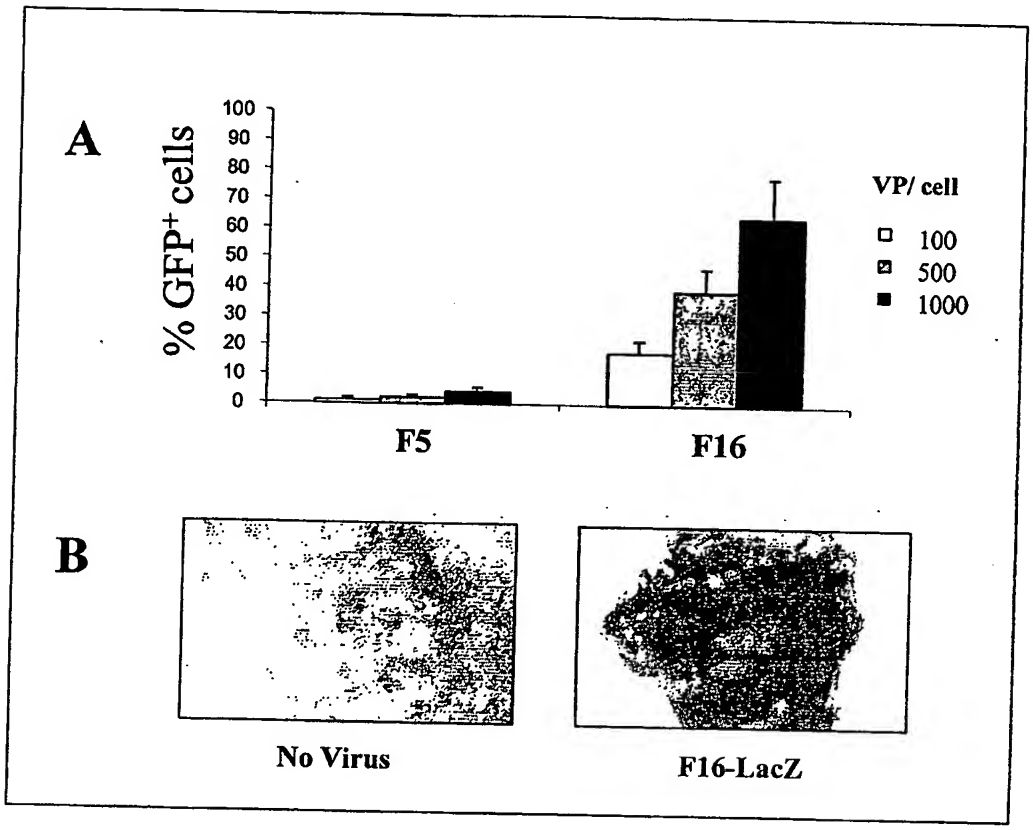
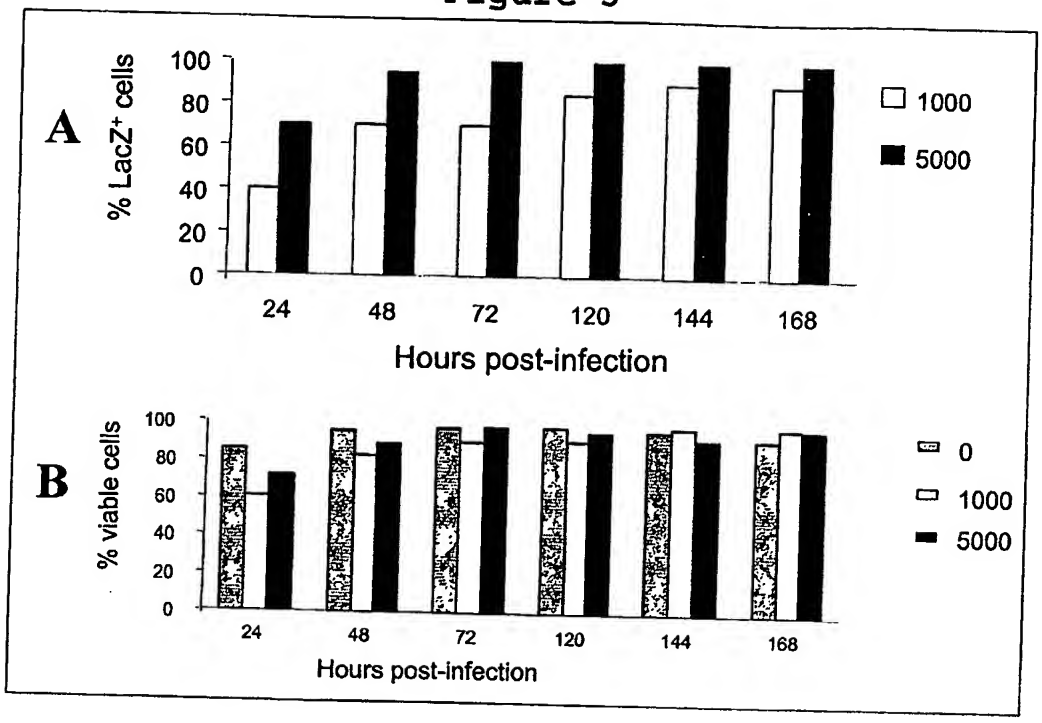

A
% GFP+ cells
100
90
80
70
60
50
40
30
20
10
0
F5
F16
VP/ cell
100
500
1000
B
No Virus
F16-LacZ

Figure 3


A
% LacZ+ cells
100
80
60
40
20
0
24
48
72
120
144
168
Hours post-infection
1000
5000
B
% viable cells
100
80
60
40
20
0
24
48
72
120
144
168
Hours post-infection
0
1000
5000

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 01 20 2619

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	HAVENGA, M.J.E. ET AL.: "Improved adenovirus vectors for infection of cardiovascular tissue" JOURNAL OF VIROLOGY, vol. 75, no. 7, April 2001 (2001-04), pages 3335-3342, XP002198345 * page 3336, column 1, paragraphs 3,4; figures 1,2 *	1-14,17, 22,23	C12N15/86 A61K48/00 C12N5/10
X	& EP 1 020 529 A (INTROGENE BV) 19 July 2000 (2000-07-19) * page 9, line 10 - line 39 * * page 11, line 1 - line 35 * * page 13, line 52 - line 54 * * page 15, line 36 - line 47 *	1-37	
X	WO 00 52186 A (SCHOUTEN GOVERT JOHAN ;VOGELS RONALD (NL); BOUT ABRAHAM (NL); INTR) 8 September 2000 (2000-09-08) * page 12 - page 35 *	1-24,27, 29,30,36	
	-/--		

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

C12N

INCOMPLETE SEARCH

The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

Although claim 25 is directed to a method of treatment of the human/animal body (Article 52(4) EPC), the search has been carried out and based on the alleged effects of the compound/composition.

Place of search	Date of completion of the search	Examiner
MUNICH	7 May 2002	Alt, G

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C07)

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PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 01 20 2619

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	MARX J C ET AL: "HIGH-EFFICIENCY TRANSDUCTION AND LONG-TERM GENE EXPRESSION WITH A MURINE STEM CELL RETROVIRAL VECTOR ENCODING THE GREEN FLUORESCENT PROTEIN IN HUMAN MARROW STROMAL CELLS" HUMAN GENE THERAPY, XX, XX, vol. 10, no. 7, 1 May 1999 (1999-05-01), pages 1163-1173, XP000982448 ISSN: 1043-0342 * page 1167, column 1 - column 2 * ---	1,2	
X	CONGET P A ET AL: "ADENOVIRAL-MEDIATED GENE TRANSFER INTO EX VIVO EXPANDED HUMAN BONE MARROW MESENCHYMAL PROGENITOR CELLS" EXPERIMENTAL HEMATOLOGY, NEW YORK, NY, US, vol. 28, 2000, pages 382-390, XP002949922 ISSN: 0301-472X * page 384, column 1, paragraph 1 *	1,3,11, 14,17, 26,37	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
Y	* the whole document * ---	1-24, 26-37	
X	TURGEMAN G ET AL: "BONE STEM CELL MEDIATED GENE THERAPY AND TISSUE ENGINEERING" JOURNAL OF BONE AND MINERAL RESEARCH, NEW YORK, NY, US, vol. 15, no. SUPPL 7, September 2000 (2000-09), page S196 XP001055433 ISSN: 0884-0431	1,3,11, 14,17, 24-26, 31-34,37	
Y	* abstract * --- -/--	1-37	

EPO FORM 1503 03.82 (P04C10)

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PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 01 20 2619

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
Y	ROELVINK PETER W ET AL: "Identification of a conserved receptor-binding site on the fiber proteins of CAR-recognizing adenoviridae" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, US, vol. 286, no. 5444, 19 November 1999 (1999-11-19), pages 1568-1571, XP002193668 ISSN: 0036-8075 * the whole document *	1-37	
Y	ROELVINK P ET AL: "The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F" JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 72, no. 10, October 1998 (1998-10), pages 7909-7915, XP002111071 ISSN: 0022-538X * the whole document *	1-37	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
A	EP 1 067 188 A (INTROGENE BV) 10 January 2001 (2001-01-10) * whole document, especially figure 10 *	1	

EPO FORM 1503 03.82 (P04C10)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 01 20 2619

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
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07-05-2002

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			WO	0104334 A2	18-01-2001

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